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ORIGINAL ARTICLE

Isolation, Purification, Characterization and Immunomodulatory activity of isolated flavonoid fractions derived from *Abelmoschus ficulneus* L. Wight & Arn.: Preclinical studies

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ABSTRACT
Many of today's diseases are caused by immunological mechanism imbalances. This necessitates the creation of new immunomodulators that are both more powerful and safer. The Indian Ayurvedic Pharmacopoeia recommends the use of A. ficulneus L.herb to treat a variety of medicinal problems ingout, polyuria, haemorrhagic, bleeding piles andblood diseases. On the basis of folkloric uses, A. ficulneus L.was selected for the exploration of immunomodulatory potential. Based on the findings study proposed to evaluate the immunomodulatory activity of isolated leaves flavonoidfractions of A. ficulneus L. Column chromatography were used to separate and purify the leaves extracts by fouriertransform infrared spectrometer (FTIR), high-performance thin layer chromatography (HPTLC), and high-performance liquid chromatography methods (HPLC).were used to characterise it. Acute oral toxicity, effect on cytokine expressions, Interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α),Interleukin (IL-4) by ELISA, phagocytosis by macrophages, and nitrite content (macrophage) development by in vitro method were all evaluated for isolated flavonoid fractions from ethanolic leaves extract. In vivo and in vitro findings were astounding, with isolated flavonoid fractions having a strong immunostimulatory impact. Macrophage phagocytosis and production of nitric oxide were significantly improved (P <0.05) when isolated fractions were given at doses of 100 mg/kg and 200 mg/kg. Furthermore, isolated fractions stimulated cytokine secretion, including IFN-γ, TNF-α, and IL-4, in a substantial (P< 0.05) way. The results shows that isolated flavonoid fractions of A. ficulneus L. possesses strong immunostimulatory activity, which supports its conventional usesfor the treatment and management of diseases that need immune system stimulation, such as infectious diseases.
Keywords: *A.ficulneus L. Immunomodulation, Macrophages, Phagocytosis, Cytokines-IFN-γ, TNF-α and IL-4.*

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INTRODUCTION

Traditional systems of medicine are used to establish immunity, to avoid infections/diseases, to prevent or relieve the effects of the disease. Simplicity of availability, cost effectiveness and assumed protection are the key factors that make natural products attractive to candidates for human use. [1]

Anti-allergic, corticosteroids, anticancer-chemotherapeutic agents, and nonsteroidal anti-inflammatory drugs (NSAIDs) were used to monitor immunological emergencies and pathogens. These medications have many side effects, however. Phyto remedies tend as immunomodulatory agents to be essential sources. The position of 'Rasayana' as a 'rejuvenation device' is recorded by Ayurveda. Therefore, exploring Phyto remedies for possible immunomodulatory function appear to be fruitful.[2]

The traditional Indian health care system, Ayurveda, is the world's oldest medical system that utilizes the potential of multiple herbs as medicines. Herbal medicines' therapeutic benefit has received a lot of attention in recent years.[3]

Many herbs are used in the indigenous system of medicine to eventuate the immune responses of body's system of the body. Phytochemicals including flavonoids, lectins, polysaccharides, tannins and peptides

were detected to attenuate the immunological responses in different *in vivo* experimental animal models.[4]

The genus Abelmoschus (Family - Malvaceae) be one of a group of flowering plants of medicinal significance. The *Abelmoschus* species owing to the being of prominent phytochemicals, *A.ficulneus*L. has a prominent role in conventional medicinal procedures. *A. ficulneus* L. is an edible medicinal plant that has a large array of medicinal uses. The whole facility is used for the ministrations of industrial applications of sprain, bronchitis, and toothache and Abelmoschus taxa.[5]. The phytoconstituents specifically flavonoids of *A. ficulneus*L. detected to be superintended for all pharmacological activities, including jaundice, a major fatal disease.[6]. Therefore, the proposed work aims to isolate, purify, characterize and screened for immunomodulatory activity of isolated flavonoids fractions of *A. ficulneus* L.

MATERIAL AND METHODS

Chemicals

Acetone, gallic acid,ethyl acetate, ethanol, methanol, acetone,quercetin and silica gel. Chemicals were used for studies are of analytical grade and were purchased fromHi media, Pvt. Ltd.and Modern Industries, Sinner, Mumbai, India.Levamisole (Khandelwal pharma. Ltd. Mumbai)

Experimental animals

In this analysis, Swiss albino mice (10–12 weeks old, 18-22 g body weight) were used to assess the immunomodulatory effect of either sex (n=6 in each group of statistically significant models) and were housed at 25^0 C ± 5^0 C in a well-ventilated and maintained animal house under the 12/12 h light/dark cycle."Total 82 Swiss albino mice were bought and used for study from LACSMI Bio Farms, # 12, "Rachana Blossom", Jagdishnagar, Aundh, Pune-411 007, Maharashtra CPCSEA Registration Number: 1277/PO/RcBt/S/09.

All mice were remained in an animal house certified according to the CPCSEA, Department of Animal Welfareand Government of India guidelines under standard conditions. The pelleted diet and distilled water *ad libitum* were provided to all mice. A protocol accepted by the Institutional Animal Ethics Committee on experimental animals (IAEC). (Protocol No. 884/PO/Re/S/05/CPCSEA).

Plant material and authentication

In July,*A. ficulneus*L. herb was collected and procured, and the leaves dried in the shade. From botanical survey of India, Maharashtra, Pune, the *A. ficulneus* L. was certified by herbarium section Pune, no. 2014/BSI/WRC/Cert./2014.

Extraction, Isolation and Fractionation of immunostimulatory flavonoids

The fresh leaves were pulverised into a coarse powder after being dried in the shade. The leaves powdered (1 kg) was defatted with petroleum ether before being extracted with ethanol using a Soxhlet extractor and then cold maceration with 50 % methanol for 7 days. To make ethanolic extract, the extracts were evaporated and concentrated using a vacuum evaporator (27.23 %). The ethanolic extract was then subjected to a column chromatography fractionation procedure.[7]

Column Chromatography

Over a silica gel column, 10 g of leaves extract of ethanolic solvent were chromatographed using solvents of increasing polarity (60-120 mesh size). The concoction was packed on a silica gel column (Merck, India), and column elution began with 100 % n-hexane, followed by changes in polarity using chloroform, ethyl acetate, ethanol, methanol in the ratios of 90:10, 80:20, 70:30, and 50:50.Ten fractions (C-1 to C-10) were collected then subjected for TLC, with identical Rf values have been pooled into such fractions based on the TLC profile.[8]

The condensed and dried fractions (C-1 to C-10) needed were stored in containers with an appropriate label and held for further use. So as to obtain a sufficient amount of isolated flavonoid fractions (IF) for the immunomodulatory activity, this procedure was repeated.[9, 10]

Thin layer chromatography (TLC)

All isolated compounds were detected by the method thin layer chromatography using silica gel 60 TLC plates. The volume of the spots applied on the chromatographic plates was 5 µl and TLC were performed by ethyl acetate: water: glacial acetic acid: formic acid (100:26:11:11) mobile phase for both extracts. The presence of flavonoid was detected by the formation of yellowish-brown colour on TLC plate by exposure of polyethylene glycol reagent on TLC plate. The chromatograms were evaluated without chemical treatment, under UV 254 and UV 365 nm light. Rf value was calculated by the formula, distance covered by the sample and distance covered by the solvent. Based on Rf values of compounds combined and labelled as C-1 and C-2 (Fig. 4) were matched with standard compound quercetin and used for further characterization by HPTLC and IR methods.[11]

High performance thin layer chromatography (HPTLC) for isolated flavonoid fractions

Chromatographic analysis carried out on plate size 10×10 cm HPTLC plates silica gel of grade 60 F_{254} purchased from E. MERCK KGaA instrument (Germany). Samples of standards and isolated fractions were applied as band length 6.0 mm wide and 8.0 mm apart by CamagLinomat 5 sample applicator. Analysis was done at 380 nm in absorbance by win CATS Chromatography software.[12]Isolated fractions of ethanolic extracts obtained by column chromatography techniques were dissolved in methanol 70% (30 mg/mL). Filtered solutions were applied to glass plates 10 cm × 10 cm (Merck, Germany). Mobile phase was used for chromatogram observation for the best resolution is ethyl acetate: water: glacial acetic acid: formic acid (100:26:11:11).Analysis was done at 380 nm in absorbance remission mode by win CATS Chromatography software.

By dissolving 10 mg of accurately weighed quercetin in 100 ml methanol, a quercetin stock (100 g/ml) solution of was prepared. 5 µl -20 µl standard solution was applied to HPTLC plate in the range 500-2000 ng per band for calibration.

According to the International Council for Harmonization (ICH, 2005) guidelines, the analytical approach was validated for precision, linearity, limit of detection (LOD), and limit of quantization (LOQ).The specificity calculated by comparing Rf values and ultraviolet-visible spectrum of peaks of components in sample and standard chromatogram.[12, 13]

Characterization of flavonoid fractions: Fourier Transformed Infrared analysis (FTIR)

For characterization of compounds IR spectra were performed. IR was performed for the isolated flavonoids of leaves extract of *A. ficulneus* L. The sample (1–2 mg) was crushed in KBrpellets (3–4 mg) using a FTIR (Model: IRAffinity-1, with Configuration of full mid-IR wave number from 7800 cm-1 to 350 cm-1, spectral resolution of 0.5 cm-1.) by mechanical pressure. Isolated fractions of flavonoids were studies in wave number range 4000–400 cm_1 in FTIR instrument.[14]

High Performance liquid chromatography

HPLC Binary Gradient System (Model no. HPLC 3000 series) was used for isolated fractions of flavonoids from herbal extract sample analysis. HPLC system is equipped with an autosampler, column compartment, pump and UV -3000- M detector. The column used for the isolated fractions of flavonoids analysis was comosilC-18 (250 mm × 4.6 ID, Particle size 5 µ). The samples concentration used were 1000 µg/ml using methanol as a solvent. The standard Quercetin concentration used was 30 µg/ml with methanol as solvent. Mobile phase used was methanol: water (75:25). Injection volume and flow rate were 20µl and 0.8 ml/min respectively. UV visible spectra were taken down over range of 200-400 nm. Chromatogram were acquired at 256 nm. Analysis was performed by HPLC workstation software.[15, 16]

Acute oral toxicity

Acute oral toxicity was carried out by 420 OECD guidelines. There were no signs of toxicity or deaths observed in mice treated by a single oral administration of the isolated fractions of *A. ficulneus* L. leaves extract at limit dose 2000 mg/kg p.o. $1/10^{th}$ of this dose i.e., 200 mg/kg for AEAF and EEAF were used in the subsequent study respectively.[17]

Assessment of immunostimulatory activity

Macrophage functional assays: Phagocytosis by macrophages:

Isolated fractions (200 mg/kg) treated mice had their peritoneal macrophages harvested by flushing the cavity with 5 ml of RPMI 1640 medium, pelleted by centrifugation at 1100 rpm for 10 minutes, and resuspended in RPMI 1640 containing 10% heat activated foetal bovine serum, 4 mM glutamine, 100 units/mL penicillin, 100 g/ml streptomycin, and100 mM sodium pyruvate. The viable cell suspension (2×10^6 cells/ml) was allowed to adhere to glass slides for 2 hrs at 37°C in a humidified CO_2 incubator. The glass slides were washed thoroughly to remove non-adherent cells. 100 µl of opsonized Candida cells (100°C, 30 min) was then spread over the adherent cells. After a thorough washing with PBS, the slides were incubated for 15 minutes in a humidified CO2 incubator and stained with trypan-eosin to assess the adherent cells containing yeast cells microscopically.[18]. The following formula was used to measure the phagocytic potential and index: PC = number of macrophages containing yeast cells / total number of macrophages counted minus 100. The Phagocytic Index (PI) is measured as the number of yeast within macrophages divided by the number of macrophages phagocytozing.[19, 20]

Macrophage nitrite content assay

On day 15, ten millilitres of RPMI-1640 were injected into the peritoneal cavity of challenged mice. The medium was removed after 5 minutes and centrifuged at 1800 rpm for 10 minutes at 4°C.In RPMI 1640 medium, the cell pellet was resuspended. In a CO_2 incubator, 3×10^6 macrophages were seeded in a 24-well culture plate for 3 hours. Non-adherent cells were removed at the end of the incubation cycle, and plates were incubated for another 48 hours in the presence of LPS (1 g/ml).[21]In a 96-well microplate, 100 µl of culture media was incubated for 10 minutes at room temperature with 150 µl of Griess reagent

(1 % sulfanilamide, 0.1 % naphthylthylene-diaminein 2.5 % phosphoric acid solution). An ELISA plate reader was used to measure the absorbance at 540 nm. The sodium nitrite norm was used to create calibration curves.[22, 23]

Cytokine expression profile study: Determination of cytokines by ELISA

Cytokine estimation was performed in the serum samples as per the recommendation of the manufacturer. Serum from blood samples was collected 4 hrs after the oral administration of isolated extracts (200 mg/kg). Interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α) and Interleukin IL-4 concentration were measured by enzyme linked immunosorbent assay by ELISA kit.[24, 25]

RESULTS

Column chromatography

To obtain fractions for yellowish brown amorphous powder, ethanol leaves extract (10 g) was subjected to column chromatography on silica gel (100-200 mesh) and eluted with mixtures of acetone, ethyl acetate, ethanol, and methanol of increasing polarity. Approximately ten fractions were eluted with increasing polarity solvents. (Fig. 1) The Rf values of column fractions 5 to 7 and 8 to 10 with ethyl acetate: ethanol (80:20) in the TLC mobile phase solvent ratio of acetone: methanol (1:1) were 0.46 and 0.47, respectively, which were identical to those of regular quercetin. (Fig. 2) The fractions were then mixed and crystallised, yielding around 100 mg in total. This procedure was repeated several times with larger samples until the correct amount of quercetin was collected.

Fig. 1: Isolated flavonoid fractions of *A. ficulneus* L.

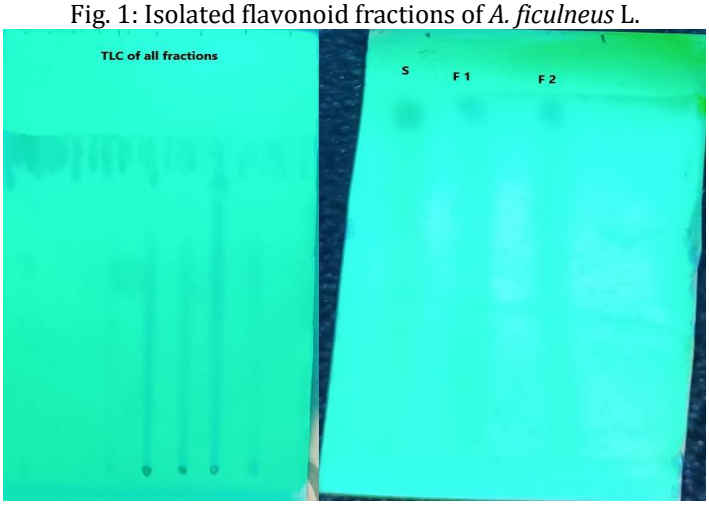

Fig. 2: Thin layer chromatography of isolated flavonoid fractions of *A. ficulneus* L.

High performance thin layer chromatography

Preparation of calibration curve and linearity

The standard solutions of quercetin were prepared (100 µg/ml) in methanol. They were diluted to make different concentrations for calibration curves and were applied on TLC plate.The plate was scanned (380 nm) and curve were plotted with respect to peak area vs. concentration/amount per spot. (Fig. 3). A good linearity was calculated with correlation coefficient (r2) value of 0.9831 for quercetin (Table 1).

Table 1: Parameters for method validation for the quantification of quercetin

Parameters	Results
Range of linearity (ng/band)	1000-35000
Regression of equation	1329.4x + 4708.4
Slope	1329.4
Correlation coefficient (r2)	0.9831
LOD (ng/band)	8.3787
LOQ (ng/band)	25.39

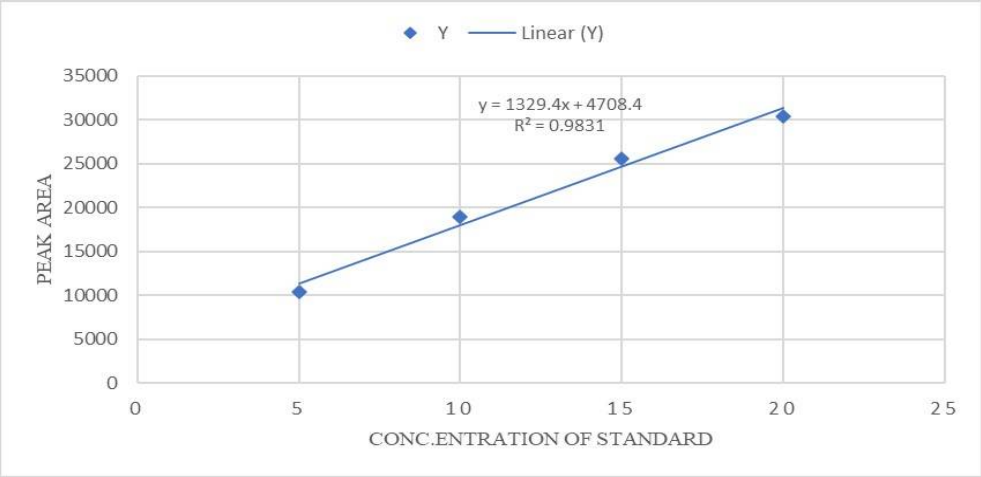

Fig. 3: Calibration curve of standard quercetin

Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ were calculated based on standard deviation (SD) and slope (S) of the calibration curve at levels according to formula {LOD = 3.3 (SD/S) and LOQ = 10 (SD/S)}. and found to be 8.3787, 25.39 ng for quercetin (Table 1).[26]

Specificity

Rf values and ultraviolet-visible spectra of component peaks in samples and a standard chromatogram were compared to determine specificity. Observation shows the Rf values at 0.64 and 0.62 at 380 nm respectively (Fig. 4 and 5) for quercetin in standard and samples respectively by densitometry.

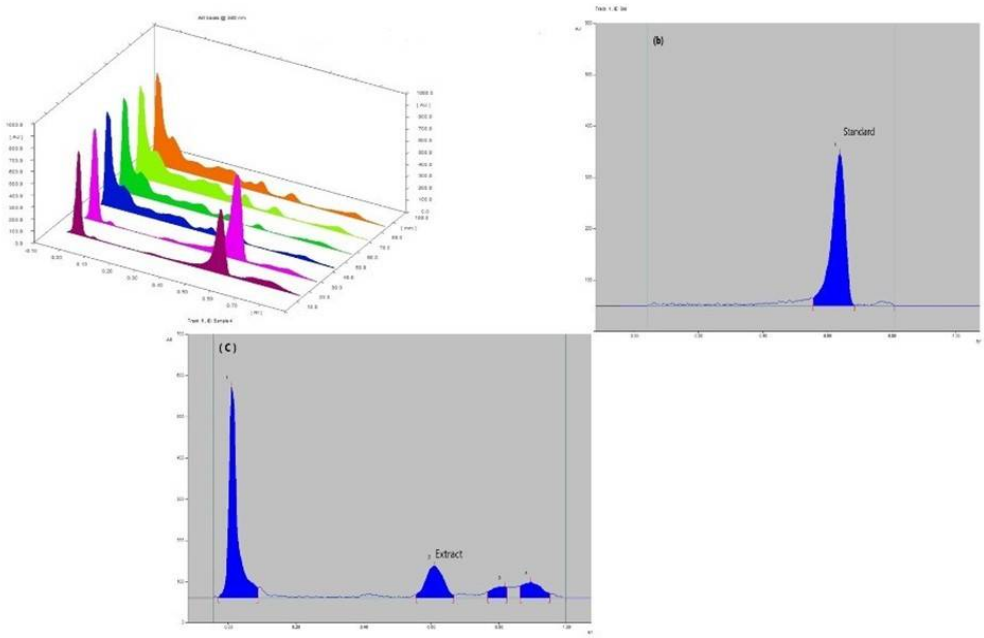

Fig. 4: HPTLC chromatogram of isolated fraction with quercetin (a) 3D display of leaf extract with quercetin chromatogram, (b) HPTLC densitogram and chromatogram of standard quercetin and (c) leaf extract at 380 nm.

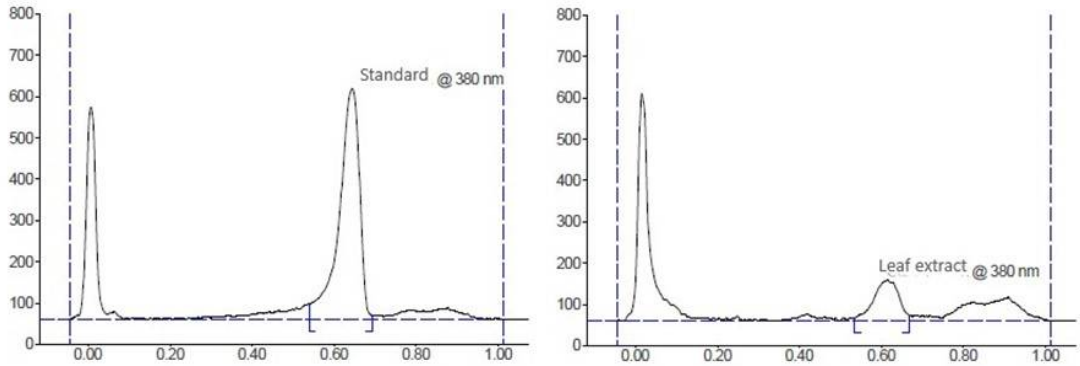

Fig. 5: HPTLC Densitogram of Standard (Quercetin) and isolated fractions*Rf* values are 0.64 and 0.62 at 380 nm respectively.

High performance liquid chromatography

HPLC qualitative analysis confirmed the results acquire by the TLC. Quercetin was found as dominating compound in the tested fraction. HPLC qualitative analysis validate the presence of Quercetin in the stem bark powder of *A. ficulneus* L.

To know the different flavonoids in the different isolated fractions of the *A. ficulneus* L. the retention times of the isolated fractions in the chromatogram were compared with the accurate standards. (Fig. 6) Quercetin used as a standard. Both the isolated fractions show the characteristic peak of flavonoids thus *A. ficulneus* L. contains the highest amount of quercetin in isolated fractions. High amount of flavonoid content of the isolated flavonoid fractions could be correlated to the present study on immunomodulation of *A. ficulneus* L. in recent literature depicted in fig. 7 and 8.

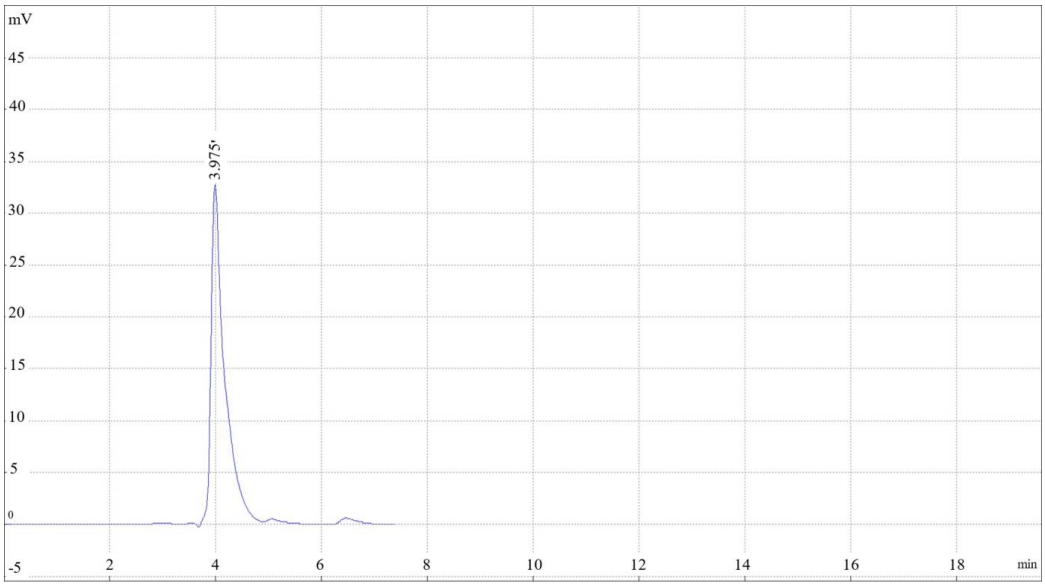

Fig 6:HPLC chromatogram of standard quercetin

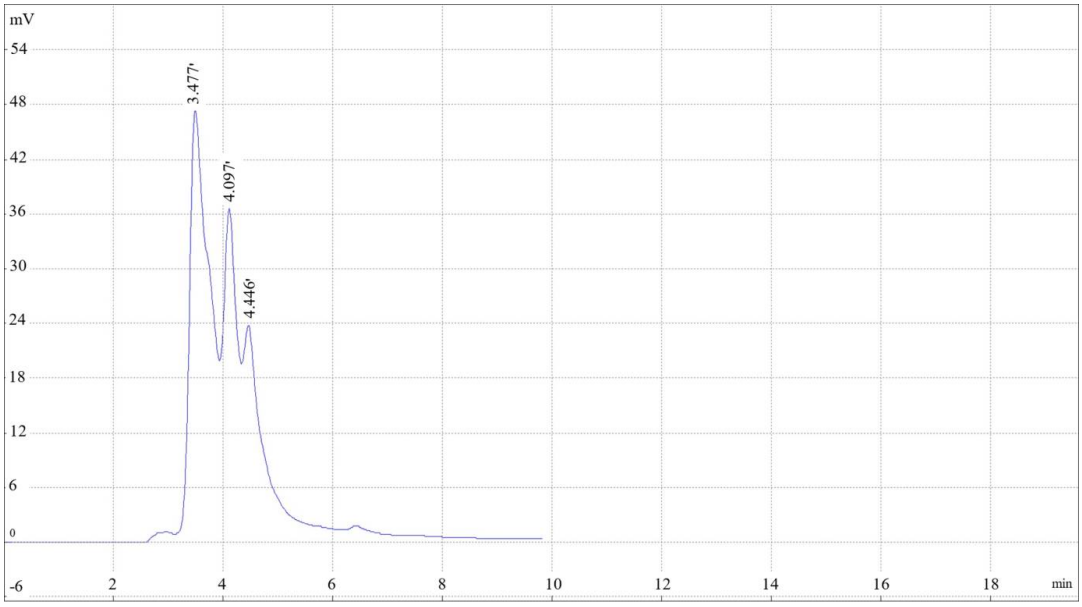

Fig 7:HPLC chromatogram of Isolated fraction 1

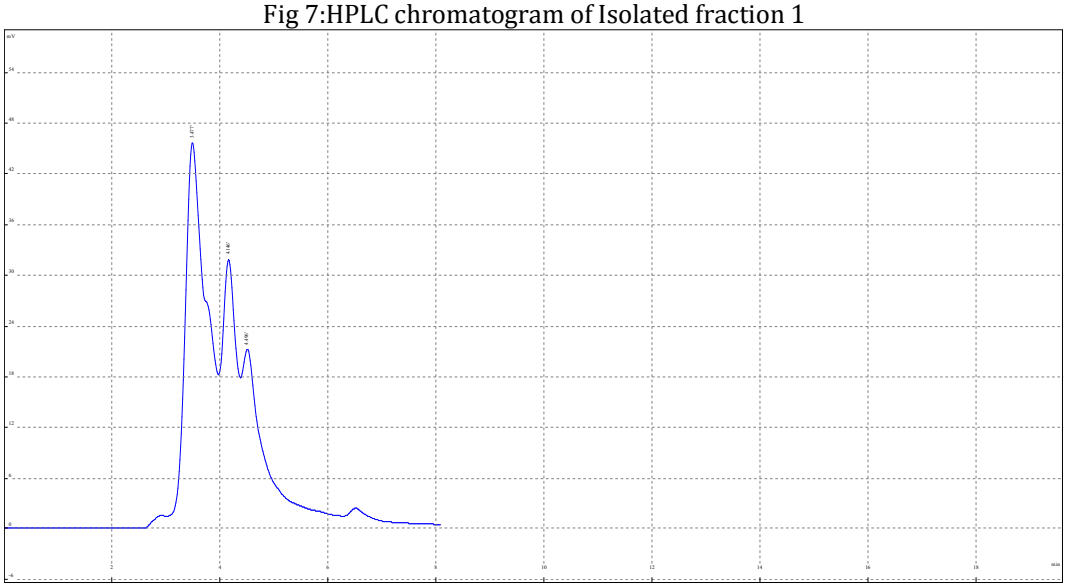

Fig 8:HPLC chromatogram of Isolated fraction 2

FT-IR

FTIR spectra of isolated fractions shown in Fig. 9 and 10. FT-IR spectroscopy provides a fingerprint of the drug through which we can identify the natureof bonding and types of functional groups in the samples. All values of IR mentioned in decreasing order. The characteristic broad peaks 3340.71 cm−1, 3255.71corresponds to the O-H hydroxyl stretching vibration. The absorption peak at 2931.80 cm−1 corresponds to the C-H stretching vibration, 2746.63 cm−1, 2360.63 cm−1, and 2059.98 cm−1 corresponds to carboxylic acid, 1705.07 cm−1, indicated the bending vibrational modes of C-O stretching in the pyranose form. In addition, the peak at 948.98 cm−1, 771.53cm−1, resulted from the presence of α-glycosidic bounds, which confirms that the isolated compound is flavonoid quercetin. The peaks showed the number of functional groups that have a major impact on *A. ficulneus* L. medicinal potential.

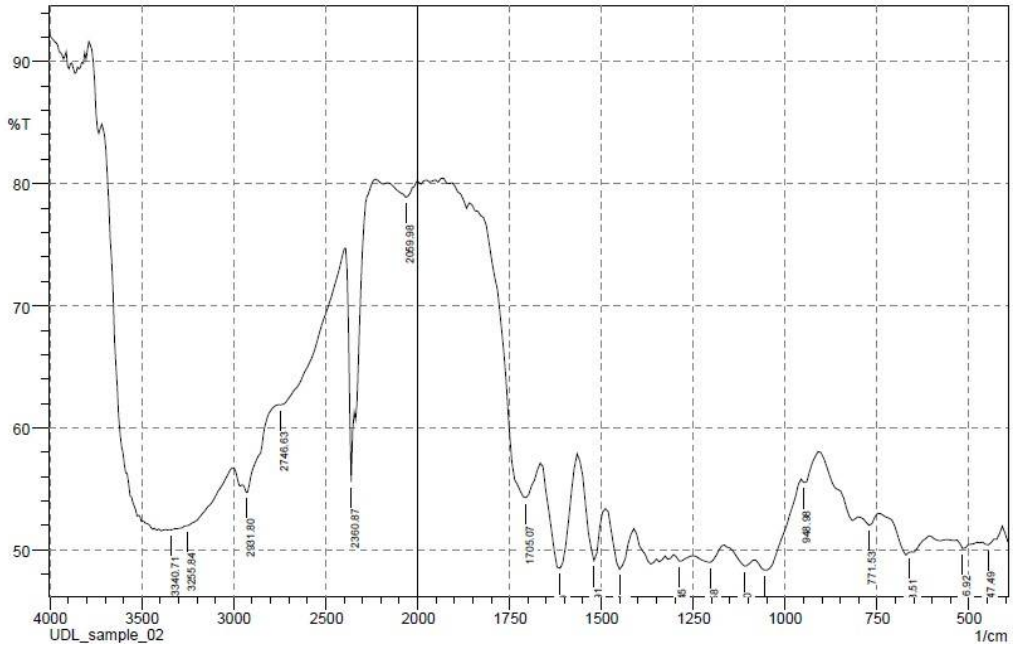

Fig.: 9: FT-IR Spectrum of IF 1

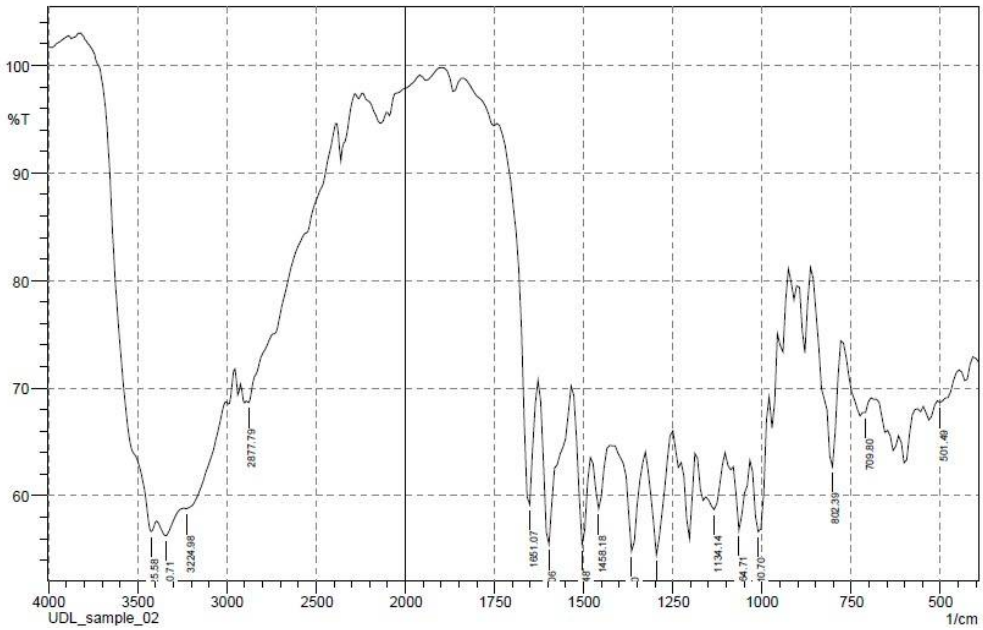

Fig.: 10: FT-IR Spectrum of IF 2

Acute oral toxicity

Acute oral toxicity was carried out by up and down regulation method. There were no signs of toxicity or deaths observed in mice treated by a single oral administration of the both leaves extract of *A. ficulneus* L.at limit dose 2000 mg/kg p.o. 1/10th of this dose i.e., 200 mg/kg for AEAF and EEAF were used in the subsequent study respectively.

Assessment of immunomodulatory activity

Macrophage functional assays: Phagocytosis by macrophages

The phagocytic feature of adherent macrophages was improved by pre-incubation with isolated fractions of *A. ficulneus* L. leaves extract.With in vivo doses ranging from 100 to 200 mg/kg relative to the control group, a large increase in the number of cells phagocytosing the yeast cells ingested per adherent cell (phagocytic index) was observed, with the highest increase occurring at the 200 mg/kg dose shown in Table 2 and Fig.11.

Table No.2: Effect of isolated flavonoid fractions of ethanolic leaves extract of *A.ficulneus* L. on Phagocytosis by macrophages.

Groups (n=6)	Treatment	Phagocytic Capacity (PC)	Phagocytic Index (PI)
Vehicle control	Distilled water, 10 ml/kg, p.o.	29.63 ± 1.4	1.27±0.04
IF 1	200 mg/kg, p.o.	51.85±1.0	1.75±0.05
IF 2	200 mg/kg, p.o.	57.40±1.0	1.83±0.06
Levamisole	LMS 50 mg/kg, p.o.	65.92±0.74	1.94±0.04

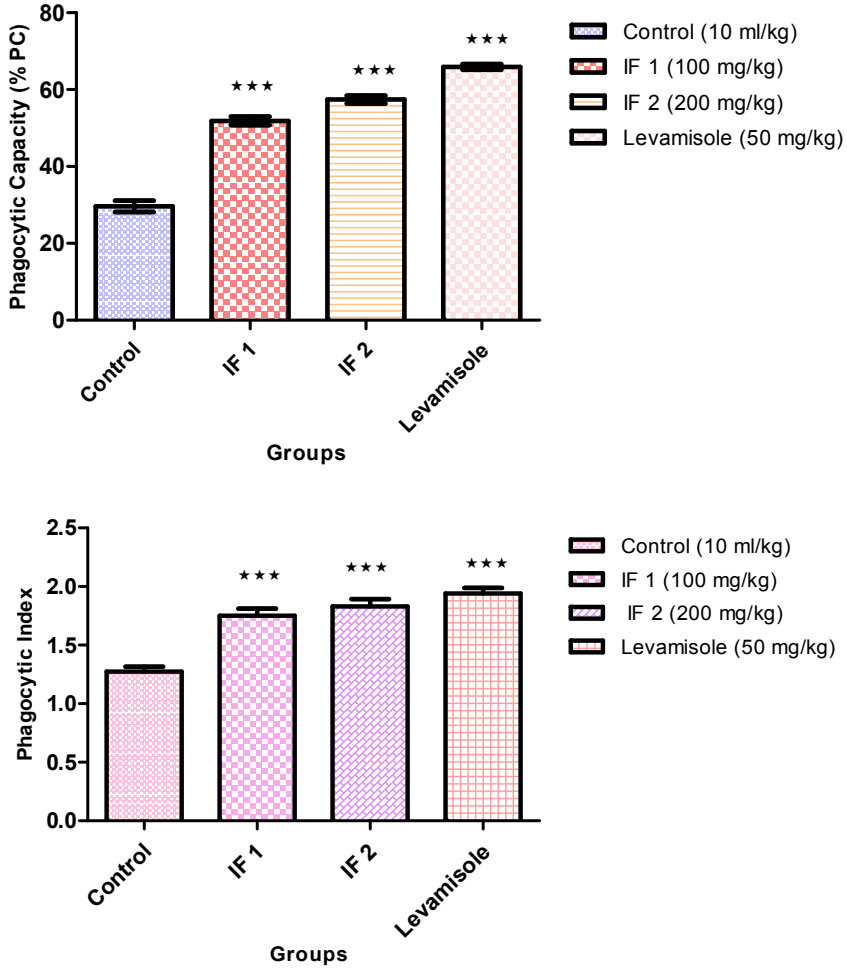

Fig. 11:Effect of isolated flavonoid fractions of *A. ficulneus* L. leaves extract (100-200 mg/kg) on Candida albicans phagocytosis.The average number of bacteria or inert particles ingested per phagocytic cell over a given time period is represented by the phagocytic index. The data represents the mean SEM of six species. * $p < 0.05$, ** $p < 0.01$ and ***$p < 0.001$ when compared to the control group (one way ANOVA) (Bonferroni correction multiple comparison test).

Macrophage nitrite content assay

Figure 12and in Table 3 depicts the effect of isolated flavonoid fractions of *A. ficulneus* L. leaves extract (100-200 mg/kg) on nitric oxide intake. Extrapolation from a sodium nitrite standard curve was used to measure the nitrite concentration, which was expressed in mgs.When compared to the control group, the maximal impact was observed at 200 mg/kg. The amount of NO and TNF-α released by peritoneal macrophages of isolated fractions treated mice was calculated to determine the effect of isolated fractions on macrophage function. The nitrite levels, a stable end-product of NO metabolism, were measured using the Griess reagent. The contents of nitrite and TNF-α were calculated in the supernatants of macrophages cultured in RPMI-FBS (10%) with LPS (1 g/mL).Extrapolation from a sodium nitrite standard curve was used to measure the nitrite concentration, which was expressed in mgs. The nitrite content in peritoneal macrophages was significantly increased with increasing doses of isolated fractions (100 and 200 mg/kg). When compared to the control group, the maximal impact was seen at 200 mg/kg.

Table No.3: Effect of isolated flavonoid fractions of *A.ficulneus* L. on Macrophage nitrite content assay.

Groups (n=6)	Treatment	Nitric oxide µM/ml
Vehicle control	Distilled water, 10 ml/kg, p.o.	18.37 ± 0.68
IF 1	200 mg/kg, p.o.	24.80 ±0.43
IF 2	200 mg/kg, p.o.	31.15 ± 0.26
Levamisole	LMS 50 mg/kg, p.o.	44.42 ± 0.40

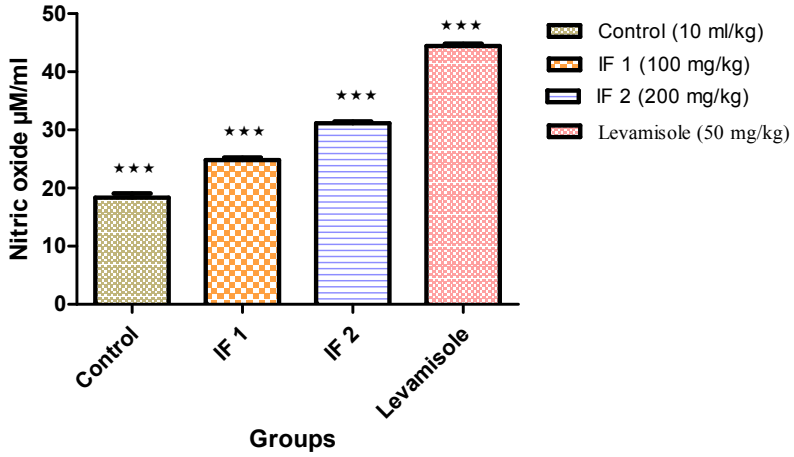

Fig. 12: Effect of isolated flavonoid fractions of *A. ficulneus* L leaves extract(100-200 mg/kg) on nitrite content in macrophages. Peritonealmacrophages were isolated from mice given graded doses of isolated flavonoid fractions of *A. ficulneus* L for14 days. Macrophages (3×10^6 cells/well) were cultured and stimulated with LPS(1 µg/mL) for 48 hrs. The supernatants were used for nitrite assay using Griess reagent.Results are expressed in µM. Data are mean±S.E.M. of six animals. * $p < 0.05$, ** $p < 0.01$ and ***$p < 0.001$ compared with control group determined by one-way ANOVA (Bonferronicorrection multiple comparison test). For experimental details refer to Materials andmethods section.

Cytokine expression profile study: Determination of cytokines by ELISA

At doses of 100 and 200 mg/kg, the isolated flavonoid fractions of *A. ficulneus* L. leaves extract produced a significant increase in the levels of IFN-γ, TNF-α and IL-4 compared with the control group shown in Fig. 13 and Table 4. In order to establish that Th1 /Th2 cytokines were involved in the immunostimulatory activity of isolated flavonoid fractions of *A. ficulneus* L. leaves extract, cytokine secretion patterns were analysed in the sera of immunized mice. isolated fraction flavonoid fractions of *A. ficulneus* L. leaves extract caused a significant (***$p < 0.001$) dose dependent equivalently increase in the Th1 (IFN-γ, TNF-α) and Th2 (IL-4) cytokines at the both doses were recorded and maximum response being at 200 mg/kg dose compared with the control group (Fig. 13 a, b and c).

Table No.4: Effect of isolated flavonoid fractions of *A.ficulneus* L. on IFN-γ, TNF-α and IL-4 cytokines determination.

Groups (n=6)	Treatment	Interferon-gamma (IFN-γ) (pg/ml) Mean ± SEM	Tumor necrosis factor-alpha (TNF-α) (pg/ml) Mean ± SEM	Interleukin IL-4 (pg/ml) Mean ± SEM
Vehicle control	Distilled water, 10 ml/kg, p.o.	23.27 ± 0.57	16.53 ±0.45	27.10±0.69
IF 1	200 mg/kg, p.o.	38.01 ± 0.47	25.08 ±0.27	35.96 ±0.44
IF 2	200 mg/kg, p.o.	38.01 ±0.42	27.57 ±0.30	37.91 ±0.28
Levamisole	LMS 50 mg/kg, p.o.	40.91 ±0.33	29.20 ±0.25	39.91 ±0.29

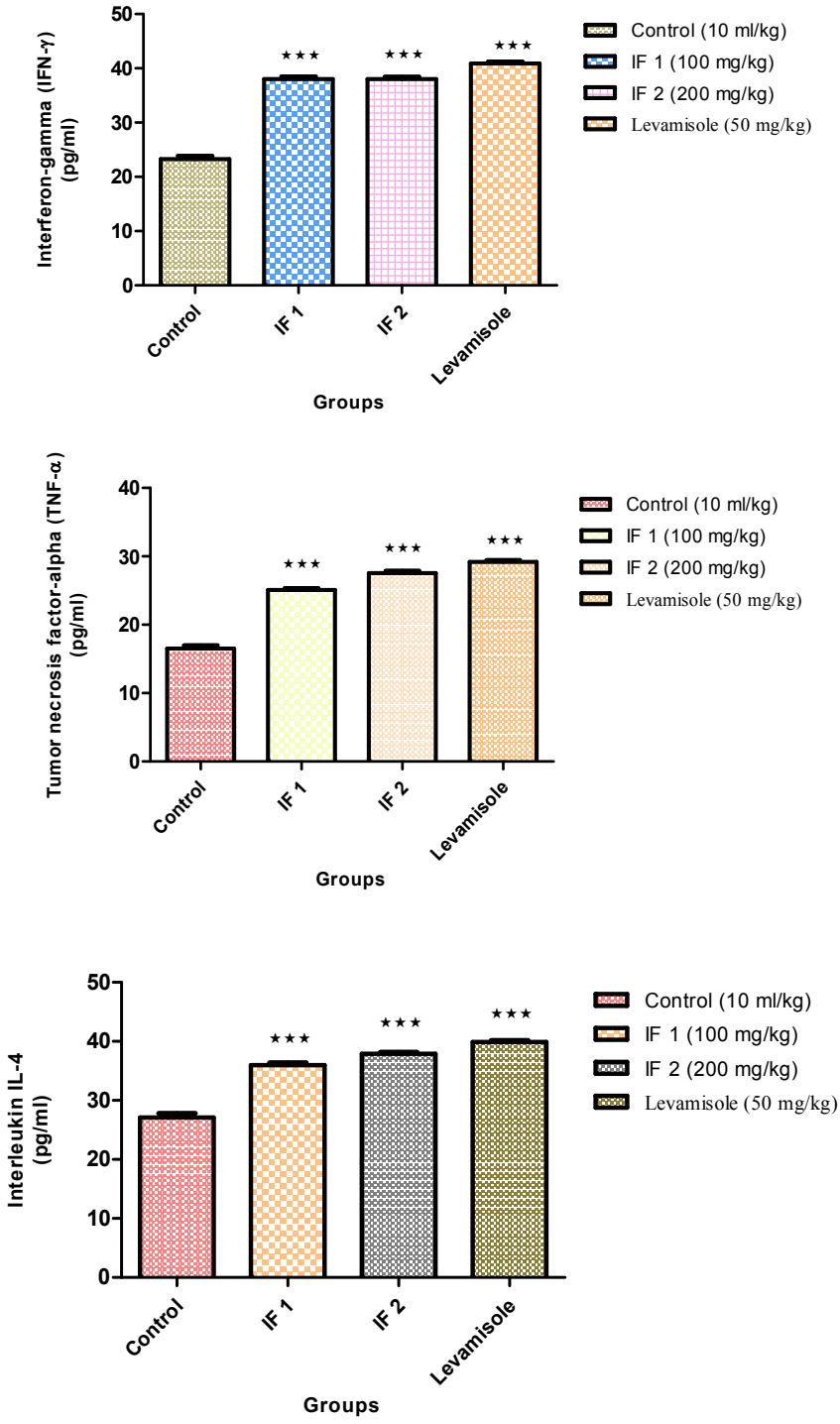

Fig. 13**:** a. Effect of flavonoid fraction on IFN-γ production in mice serumb. Effect of flavonoid fraction on TNF-αproduction in mice serum. c. Effect of flavonoid fraction on IL-4 production in mice serum, collected4 h after the final oral administration of flavonoid fraction at doses100 and 200 mg/kg. Data are mean±S.E.M. of six animals. * $p < 0.05$, ** $p < 0.01$ and ***$p < 0.001$ compared with control group determined byone-way ANOVA Bonferroni correction multiple comparison test.

DISCUSSION

Immunity is essential for protecting the human body from foreign particles. Many Ayurvedic plants have traditionally been used to improve immunity. By stimulating the nonspecific immune system, immunomodulatory agents from both origin(plant and animal) increase the body's immune

responsiveness to foreign particulate matter. However, systemic research on medicinal plants are required to back up the beneficial claims made about their clinical efficacy.[27]
Flavonoids have been shown to increase helper T cells, interleukin-2, interferon, and macrophages, making them useful in a variety of immune-related diseases.[20]
Isolated flavonoids, IR spectra confirmed the existence of the OH group in both free and bound states, which serves as an enzyme binding site, resulting in immunomodulatory effects.
Acute toxicity studies of isolated fractions showed an estimatedLD_{50} greater than 2000 mg/kg, which is an indication ofrelative safety and outlying risk of acute intoxication.
Macrophage phagocytosis is an important marker in the immunomodulation process. In vivo and in vitro, macrophages' primary function is to engulf foreign particulates. Phagocytic activity strengthens the body's immune system by serving as a second line of protection against pathogenic invasion..[28]
By phagocytosis of *C. albicans* cells and macrophage functional assays, isolated flavonoid fractions of *A. ficulneus* L. significantly increased in phagocytic ability and phagocytic index. This was followed by immunostimulatory effects by activation of non-specific immune response.
Nitric oxide (NO) is a essential signalling and effector molecule released by activated macrophages that plays an very functional role in pathogenic defence and apoptosis regulation.[21]
Since NO can aid macrophage-mediated target cell lysis and phagocytosis, measuring NO levels can provide useful information about the immunomodulatory properties of isolated flavonoid fractions of *A. ficulneus* L. IF 1 and IF 2 display a substantial increase in macrophage NO development, which protects against bacterial infection.[22]
The nature of the helper T lymphocyte (Th) subsets that participate in immune responses to protein antigens has a big impact.[29]
Th1 cells contain IFN-γ and TNF-α, which are primarily involved in cellular immunity. Th2 cells contain cytokines like IL-4 and promote the development of IgM and IgG antibodies, which is the basis of humoral immunity. The Th1/Th2 dichotomy offers a basis for comparing various forms of immune responses. This categorization of immune responses, and also has resulted in an oversimplification of autoimmune disorders into Th1 or Th2 forms. It is widely accepted as a viable therapeutic alternative. Therefore, the present need is to develop an immunomodulating agent capable of modifying the immune response. [30]
The Th1/Th2 cytokine balance should aid in recognizing the outcomes of different immune responses as well as being clinically useful in treating immunologically dysregulated states..[29]
Cytokines are generally recognised as essential regulators of the immune response to antigens and infectious agents. Th1 cells produce both IL-2 and IFN-γ, while Th2 cells only produce IL-4. The augmentation of T and B cells with isolated fractions may be due to a cytokine-mediated mechanism.IFN--γ is an essential immunoregulatory molecule that defends against viral infections, induces T cell generation, activates macrophages, and regulates crossTh1 and Th2 cells because it is upregulated in isolated fractions treated classes. Both TNF-α and IFN-γ have the potential to improve immunoregulatory function. IL-4, has ability to activate monocytes and macrophages, is synthesized by T helper(Th2) cells or mast cells. The balance of Th1 and Th2 cytokine synthesis should be useful in comprehension the consequence of various immune responses and in treating immunologically unmanaged states clinically. Isolated fractions increased IL-4 development while also releasing large amounts of IFN--γ and TNF-α, balancing the Th1 and Th2 responses.[31]
Isolated fractions had immunomodulatory activity comparable to levamisole, a normal immunomodulatory agent. Levamisole, is an immunostimulant, has the ability to restore immune function in B-lymphocytes, T-lymphocytes, monocytes, and macrophages that have been compromised.[32]

CONCLUSION

The isolated flavonoid fractions of *A. ficulneus* L. frolic to an important role in the regulation of the immune response, hence may have implementation in resisting various life-threatening infections, according to proposed work. As a result, it may be a first-line treatment for diseases characterised by T-cell and B-cell deficiency, as well as phagocytic dysfunction. Taking all of these studies into account, we concluded that the isolated flavonoid fractions of *A. ficulneus* L. tend to be dynamic modulators of innate immunity immune response.

ABBREVIATIONS

A. ficulneus L.:*Abelmoschus ficulneus* L.
IF: Isolated fractions
FTIR: Fourier Transform Infrared Spectrometer
HPTLC: High Performance Thin Layer Chromatography

HPLC: high-performance liquid chromatography methods
IFN-γ: Interferon-gamma
TNF-α:Tumour necrosis factor-alpha
IL-4: Interleukin-4
ELISA: Enzyme-linked immunosorbent assay
C. albicans: Candida albicans
PI: Phagocytic Index
PC: Phagocytic Capacity
LOD: Limit of detection
LOQ:Limit of quantitation

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